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PERI-TYRRHENIAN PHYLOGEOGRAPHY IN THE LAND SNAIL *SOLATOPUPA GUIDONI* (PULMONATA)

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ABSTRACT

The land snail *Solatopupa guidoni* has an interesting disjunct distribution in the peri-Tyrrhenian area, with a few scattered populations in Sardinia, Corsica, and Elba Island. The species is strictly bound to limestone and has poor dispersal capacity. Here, we used sequences of two mitochondrial genes, cytochrome oxidase I (COI) and 16S rRNA (16S), to reconstruct the species phylogeography. The study is based on nine of the 13 populations reported in the literature. Phylogenetic methods and nested clade analysis failed to retrieve reciprocally monophyletic lineages on each island. Sardinian and Elba Island populations were found to be embedded within those of Corsica. These results contrast with previous phylogeographic studies of other organisms with similar distribution patterns, showing major phylogeographic breaks between islands. Molecular clock estimates suggest that species diversification occurred less than 2.5 Myr ago, in line with a previous study that dated the split of *S. guidoni* from its continental sister species at 3–5 Myr. Corsican populations can be tentatively sorted into two haplo-groups. A first group includes haplotypes found in central inland Corsica only, whereas haplotypes found in northern coastal locations cluster in a second haplo-group. The latter also includes Sardinian and Elba Island populations. Molecular data indicate that a combination of vicariance and dispersal events provide the best explanation of the distribution of this species.

Key words: *Solatopupa*, land snail, phylogeography, COI, 16S, Nested Clade Phylogeographical Analysis, molecular clock.

INTRODUCTION

The genus *Solatopupa* Pilsbry, 1917, comprises a small group of six land snail species with a peri-Tyrrhenian distribution, ranging from northern Spain to central Italy, including Sardinia, Corsica, and Elba Island. All but one species (*S. cianensis*) ascribed to the genus are strictly bound to limestone. These characteristics, coupled with the general low potential for long-distance dispersal of land snails, make *Solatopupa* a reliable model for biogeographic studies. The distribution of the genus has been traditionally explained by vicariance due to paleogeographic events that caused the detachment of the Sardinia-Corsica microplate from the Iberian plate in the early Oligocene, its subsequent rotation anti-clockwise towards the Italian peninsula (early to middle Miocene), and finally its separation into two islands (Alvarez, 1972; Bellon et al., 1977; Boccaletti et al., 1990; Carmignani et al., 1995; Gelabert et al., 2002; Bosellini, 2005). The interaction of the microplate with the emerging Apennine chain during the Pliocene has been invoked to explain the occurrence of a single species, *Solatopupa guidoni* (Caziot, 1902), in Sardinia, Corsica, and Elba Island (Giusti, 1977).

Ketmaier et al. (2006) produced a phylogenetic hypothesis for the genus *Solatopupa* based on nucleotide sequences of three mitochondrial and one nuclear gene, showing that phylogenetic relationships were consistent with the paleogeography of the peri-Tyrrhenian area. However, contrary to previous reports on other taxa with similar peri-Tyrrhenian distributions, molecular clock estimates suggested that the diversification of *Solatopupa* coincided with the very last phases of detachment of the microplate (late Miocene-middle Pliocene) rather than with the initial ones (late Oligocene-early Miocene). Molecular data also suggested that

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the relatively widespread *S. similis* (Bruguière, 1792), occurring from northern Spain to eastern Liguria, Italy, is the continental sister species to the exclusively insular *S. guidoni*. Ketmaier et al. (2006) analyzed four populations of *S. guidoni* (two from Corsica, one from Sardinia and one from Elba Island) and found a close affinity between the Sardinian and Elba Island populations, whereas the Corsican populations formed a single clade. Previous allozyme data on a slightly different set of populations suggested two genetically distinct lineages in the species (F_{ST} = 0.523 over 28 loci, $P < 0.05$; Boato, 1988; at that time, the name *S. simonettae* was still used to identify the species, and only later was it synonymized with *S. guidoni*). One group comprised two populations from inland Corsica and one included two populations from northern coastal Corsica and one population from Sardinia.

Solatopupa guidoni has a very scattered distribution, with nine populations known from Corsica, two from Sardinia and two from Elba Island (Fig. 1; Giusti, 1977). Populations are strictly associated with calcareous rocks; these are of different ages in the case of Corsica and Sardinia (Fig. 1). For this study, we considerably expanded the sampling in terms of populations and individuals, and we sampled nine out of the 13 known populations and surveyed a total of 60 individuals. We could not find any individual in four other localities from which the species has been reported in the past (Fig. 1). We also surveyed other ecologically suitable areas (i.e. with limestone), particularly in central and southern Corsica but without success. It therefore seems likely that we covered the whole range of the species.

We used fragments of two mitochondrial DNA (mtDNA) genes – cytochrome oxidase I (COI) and 16S rRNA (16S) – to produce a phylogeographic hypothesis for *S. guidoni*. COI and 16S are the most variable genes sequenced by Ketmaier et al. (2006); we therefore expected to find sufficient genetic variation to clarify relationships at population level. We aimed to determine (1) whether mtDNA lineages occurring on the three islands are reciprocally monophyletic as expected in light of their allopatric distribution and the poor dispersal capacity of the species. We also want to (2) obtain an approximate temporal framework for the intraspecific diversification of the species. Because the split between *S. guidoni* and its closest continental relative *S. similis* has been dated at 3–5 Myr (Ketmaier et al., 2006), divergence in *S. guidoni* should be more recent. Finally, we aim to exploit our wider sampling to (3) test the hypothesis of Boato (1988) that in Corsica *S. guidoni* would comprise two genetically distinct lineages. We discuss our findings in light of the paleogeography of the area and phylogeographic studies conducted on a variety of groups with peri-Tyrrhenian distribution (i.e. newts, subterranean terrestrial and aquatic isopods, stoneflies, and flightless beetles) (Cobolli Sbordoni et al., 1995; Caccone et al., 1997; Ketmaier et al., 1999, 2000, 2003a, b; Fochetti et al., 2004).

MATERIALS AND METHODS

Sampling, DNA extraction, PCR Amplification and Sequencing

Five Corsican populations of *S. guidoni* were sampled, and this material was added to the four populations (one from Sardinia, two from Corsica and one from Elba Island) analyzed by Ketmaier et al. (2006). Details on sampling localities and sample sizes are given in Table 1 and Figure 1. Total genomic DNA was extracted following Ketmaier et al. (2006). We used the same primers and PCR conditions as in Ketmaier et al. (2006) to amplify about 700 and 500 base pairs (bp) of mitochondrial DNA (mtDNA) of Cytochrome Oxidase I (COI) and 16S rRNA (16S) gene fragments. PCR products were sequenced with an automated sequencer (Applied Biosystems 373A) following the manufacturer's protocols. To promote accuracy, both strands were sequenced using the primers employed for PCR. The sequences have been deposited in GenBank (Accession No. GU046364–GU046389; Table 1).

Population Genetics and Phylogenetic Analyses

Sequences were edited and aligned using Sequencher 4.6 (Gene Code Corporation); alignments were also checked by eye. The suitability of pooling sequence data from the two sequenced genes was assessed by the incongruence length difference test (Farris et al., 1994), as implemented in PAUP* 4.0β10 (Swofford, 2002). The test showed that the two regions are not phylogenetically incongruent ($P = 0.787$). We further check combinability by using the Bayesian approach detailed in Kauff & Lutzoni (2002), with a threshold value of posterior probabilities ≥ 99% for conflicting relationships. None of the few conflicting relationships revealed by the separate analyses received reciprocal pos-

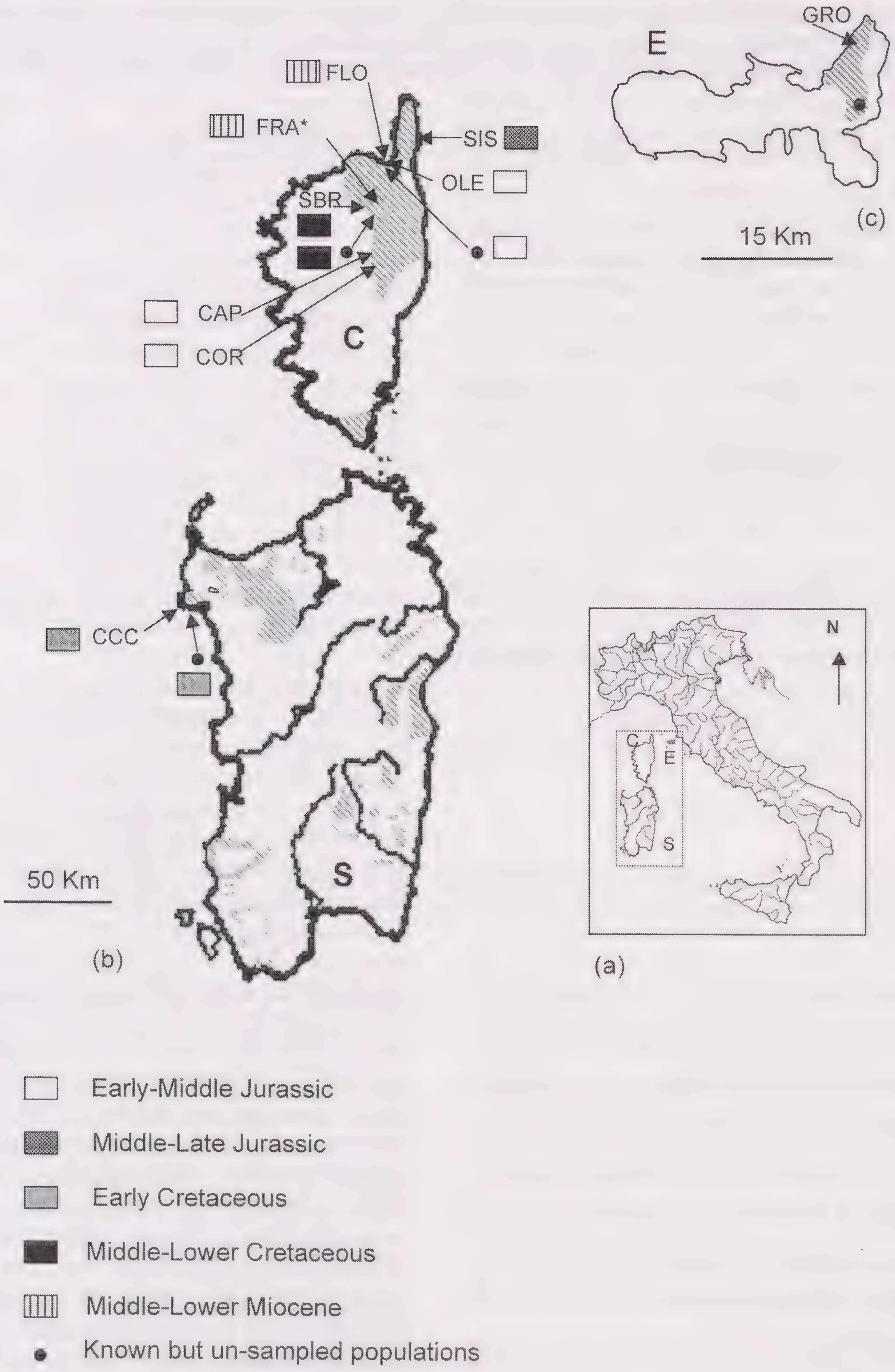

FIG. 1. Schematic maps showing the sampling localities of the *Solatopupa guidoni* populations included in the study and the geology of the region. (a) Geographic position of the three islands - Sardinia (S), Corsica (C), and Elba Island (E) - where *S. guidoni* occurs. In (b) and (c) are respectively shown sampling localities in Corsica/Sardinia and Elba Island. Three letter codes identify different populations and they correspond to those in Table 1. Black dots identify localities where the species had been reported but not found during our samplings. Downward diagonal shading indicates the distribution of limestone in three islands. Different patterned rectangles close to each population refer to the different ages of calcareous rocks inhabited by *S. guidoni* populations (this information is not available for Elba Island). Positioning of three islands roughly approximates real geographic relationships but sizes of the islands are not in proportion to one another (see scale bars).

TABLE 1. Locality, latitudinal and longitudinal data (Lat/Log) used in the nested clade analysis, sample size (N), and abbreviations for the *Solatopupa guidoni* populations included in the study. The last two columns report GenBank accession numbers. [a]Allozyme data from Boato (1988); [b]Data from Ketmaier et al. (2006).

				GenBank accession numbers	
Locality	Lat/Long	N	Code	COI	16S
Capo Caccia, Sardinia, Italy	40°33'N, 8°9'E	3	CCC[a]	DQ305079[b]	DQ305064[b]
Corte, Corsica, France	42°18'N, 9°9'E	16	COR	GU046364 GU046365	GU046381 GU046386
Sabara, Corsica, France	42°25'N, 9°8'E	4	SBR[a]	DQ305080[b]	DQ305062[b]
Francardo, Corsica, France	42°23'N, 9°11'E	3	FRA[a]	DQ305077[b]	DQ305063[b]
Caporalino, Corsica, France	42°22'N, 9°11'E	5	CAP[a]	GU046366 GU046367 GU046368 GU046369	GU046379 GU046380 GU046385
Oletta, Corsica, France	42°37'N, 9°20'E	9	OLE	GU046370 GU046371	GU046384 GU046389
St. Florent, Corsica, France	42°41'N, 9°19'E	10	FLO[a]	GU046372 GU046373 GU046374	GU046382 GU046383 GU046387
Sisco, Corsica, France	42°48'N, 9°29'E	8	SIS	GU046375 GU046376 GU046377 GU046378	GU046388
Monte Grosso, Elba Island, Italy	42°51'N, 10°24'E	2	GRO	DQ305076[b]	DQ305061[b]

terior probabilities of ≥ 99%. In light of these results, we present only analyses based on the two genes combined. We used PAUP* 4.0β10 to calculate base frequencies and to test for base frequency homogeneity across taxa (χ^2 test). To detect saturation of substitutions, we plotted the absolute number of transversions and transitions against uncorrected-p genetic distances. This was done for each gene separately and for the two genes combined. For the COI dataset, saturation plots were derived for third codon positions only.

Data was analyzed phylogenetically with *S. similis* (GenBank Accession Nos. DQ305085 and DQ305059 for COI and 16S, respectively) as outgroup. This species is sister to *S. guidoni* in the genus phylogeny (Ketmaier et al., 2006). We used MODELTEST (Posada & Crandall, 1998) to determine the model of sequence evolution that best fit our data. We then used the MODELTEST output to calculate Maximum Likelihood (ML) genetic distances for Neighbor-Joining (NJ) analyses, as implemented in PAUP. We employed the same model of sequence evolution for Bayesian searches. These were run in MrBAYES 3.0b4 (Ronquist & Huelsenbeck, 2003) allowing site-specific rate variation partitioned by gene and, for COI, by codon position. MrBAYES was run for two million generations with a sampling frequency of 100 generations. We ran one cold and three heated Markov chains and two independent runs. To assess if the Markov chains had reached stationarity, we plotted the likelihood scores of the sampled trees against generation time. Trees generated before stationarity were discarded as burn-in (first 10% of the sampled trees) and posterior probability values for each node were calculated on the basis of the remaining 90% of sampled trees. These trees were then used to construct a 50% majority rule consensus tree in PAUP. The robustness of the NJ tree was tested by 1,000 bootstrap replicates in PAUP. Phylogenetic tree topologies generated with different phylogenetic methods and competing phylogenetic hypotheses were evaluated statistically with the unbiased tree selection test (AU) as implemented in CONSEL (Shimodaira & Hasegawa, 2001). We compared tree topologies simultaneously (Shimodaira & Hasegawa, 1999).

The molecular clock hypothesis was tested with the likelihood ratio test (LRT; Goldman, 1993), which compares the log-likelihood of ML trees with and without assuming a molecular clock. Time estimates were calculated according to the formula T= $D_{ML}/2r$, where D_{ML} is the maximum likelihood genetic distance and r is the mutation rate. D_{ML} distances were calculated for the COI and 16S partitions separately using the substitution model parameters selected for each data partition with MODELTEST (Posada & Crandall, 1998). We used previous published rates for mollusks to derive divergence times; the same rates were adopted in Ketmaier et al. (2006). Thus, time estimates between that study and the current one are perfectly comparable. Rates vary between 0.67 and 1.21% per million years for COI and between 0.5 and 0.6% per million years for mitochondrial ribosomal genes (Ozawa & Okamoto, 1993; Rumbak et al., 1994; Marko, 2002).

We used the Nested Clade Phylogeographical Analysis (NCPA) (Templeton, 1998) to test for association between genealogy and geography in the dataset and to infer population processes. This method has been recently criticized, because it seems to be prone to a high rate of false positives (e.g. Knowles, 2008; Petit, 2008). However, NCPA has still a number of advantages compared to alternative methods (Templeton, 2009). In addition, we based our main conclusions on multiple independent analyses (i.e. phylogenetic and population genetics methods) to complement the NCPA inferences. We used the software ANeCA (Panchal & Beaumont, 2007), which automates the complex NCPA and renders manual procedures (i.e. the nesting design) in the analysis unnecessary. The package includes TCS, which implements the statistical parsimony procedure (Clement et al., 2000), and GEODIS (Posada et al., 2000) to respectively derive the haplotype network and to calculate distances among haplotypes and clades and their statistical significance. We included four additional locations in the GEODIS analysis (Monte Castello, Elba Island, 42°47'N, 10°22'E; Col de Teghime, Corsica, 42°40'N, 9°24'E; Sabara, Corsica, 42°24'N, 9°10'E; Punta Giglio, Sardinia, 40°34'N, 8°12'E; indicated by black dots in Fig. 1). These are localities where the species had been found in the past but not during our sampling; including this information is important to detect any sampling inadequacies (Panchal & Beaumont, 2007). The statistical distribution of distances was determined by re-calculating distances after 10,000 random permutations (i.e., clades against sampling localities). ANeCA automatically runs a slightly different version of the inference keys, dated 14 July 2004.

We used ARLEQUIN 3.0 (Excoffier et al., 2005) to calculate the following parameters of genetic diversity for each population: haplotype diversity (h), mean number of pairwise differences between all pairs of haplotypes (π) and the nucleotide diversity (π_n). Levels of genetic diversity were tested by a hierarchical analysis of molecular variance (AMOVA) (Excoffier et al., 1992) in ARLEQUIN with 1,000 replications. For this analysis we grouped populations (1) by island of provenance, and (2) by island but with Corsican populations split into two groups as suggested by the phylogeographic and NCPA results. We also used ARLEQUIN to calculate pairwise F_{ST} values for all pairs of populations; statistical significance of F_{ST} values was assessed by 1,000 permutations with sequential Bonferroni correction for multiple tests.

RESULTS

Sequence Variation

We sequenced 1,143 bp of mtDNA (675 bp for the COI gene and 468 bp for the 16S rRNA gene) for each individual included in the study. We observed only few gaps in the final alignment all in the 16S gene and confined to outgroup vs. ingroup comparisons. Levels of sequence variability were slightly higher in COI than in 16S; there were 50 (7.40%) polymorphic sites in COI and 33 (7.05%) in 16S; parsimony informative sites were 35 (5.18%) and 10 (2.13%) for the two genes. As expected, most of the variation resided in COI 3rd codon positions; 43 (19.11%) sites were variable, and of these 31 (13.7%) were also parsimony informative. The combined data set had 83 (7.26%) and 45 (3.93%) variable and parsimony informative sites. Sequences were generally A+T rich and anti-G biased, a typical pattern for mitochondrial genomes. A+T percentages ranged from 57.5% in COI 1st codon positions to 88.9% in COI 3rd codon positions; G percentages varied from 5.3% in COI 3rd codon positions to 27.9% in COI 1st codon positions. A χ^2 test of homogeneity of base frequencies among taxa did not reveal any significant differences, irrespective of the data partition tested (both genes combined,

TABLE 2. Haplotype frequencies of all individuals of *Solatopupa guidoni* sequenced for the study. For population codes see Fig. 1. Numbers in brackets are sample sizes.

	Populations								
	Sardinia			Corsica					Elba Is.
Haplotype	CCC (3)	COR (16)	SBR (4)	FRA (3)	CAP (5)	OLE (9)	FLO (10)	SIS (8)	GRO (2)
1					1				
2					1				
3					1				
4					1				
5					1				
6	3								
7		14							
8		1							
9		1							
10							8		
11							1		
12							1		
13				3					
14									2
15						8			
16						1			
17			4						
18								1	
19								4	
20								2	
21								1	

each gene separately and COI 1st, 2nd, and 3rd codon positions separately). Visual inspection of saturation plots (not shown) revealed that saturation is not present in the data set.

Haplotype Frequencies and Genetic Variability of Populations

The 60 sequences yielded 21 haplotypes (Table 2). These were population-specific (i.e., not shared among populations) and differed by 1 to 23 substitutions. COR, CAP, OLE, FLO and SIS had more than one haplotype with a maximum number of five in CAP (Table 2). CCC, SBR, FRA and GRO were fixed for single haplotypes. In the other populations, haplotype diversity (*h*) ranged between 0.222 ± 0.166 (OLE) and 1.000 ± 0.126 (CAP); the mean number of pairwise differences between all pairs of haplotypes (π) and nucleotide diversity (π_n) was lowest in COR (0.375 ± 0.376 and 0.0003 ± 0.0003) and highest in OLE (4.666 ± 2.523 and 0.004 ± 0.002).

Phylogenetic Analyses and Divergence Times

Figure 2 shows the haplotype Bayesian tree obtained for the combined data set using the GTR + I model (proportion of invariable sites = 0.876, equal distribution of rates at variable sites) and summarizes the results of the NJ searches. Both trees were statistically indistinguishable with the AU test (*P* = 0.641) and identified two major haplotype groups. A first group received good bootstrap support (89%) but fair posterior probability (89%); this group included haplotypes from Sardinia, Corsica, and Elba Island. Relationships within this clade were generally supported. Haplotypes from two Corsican localities (FLO and OLE) did not form

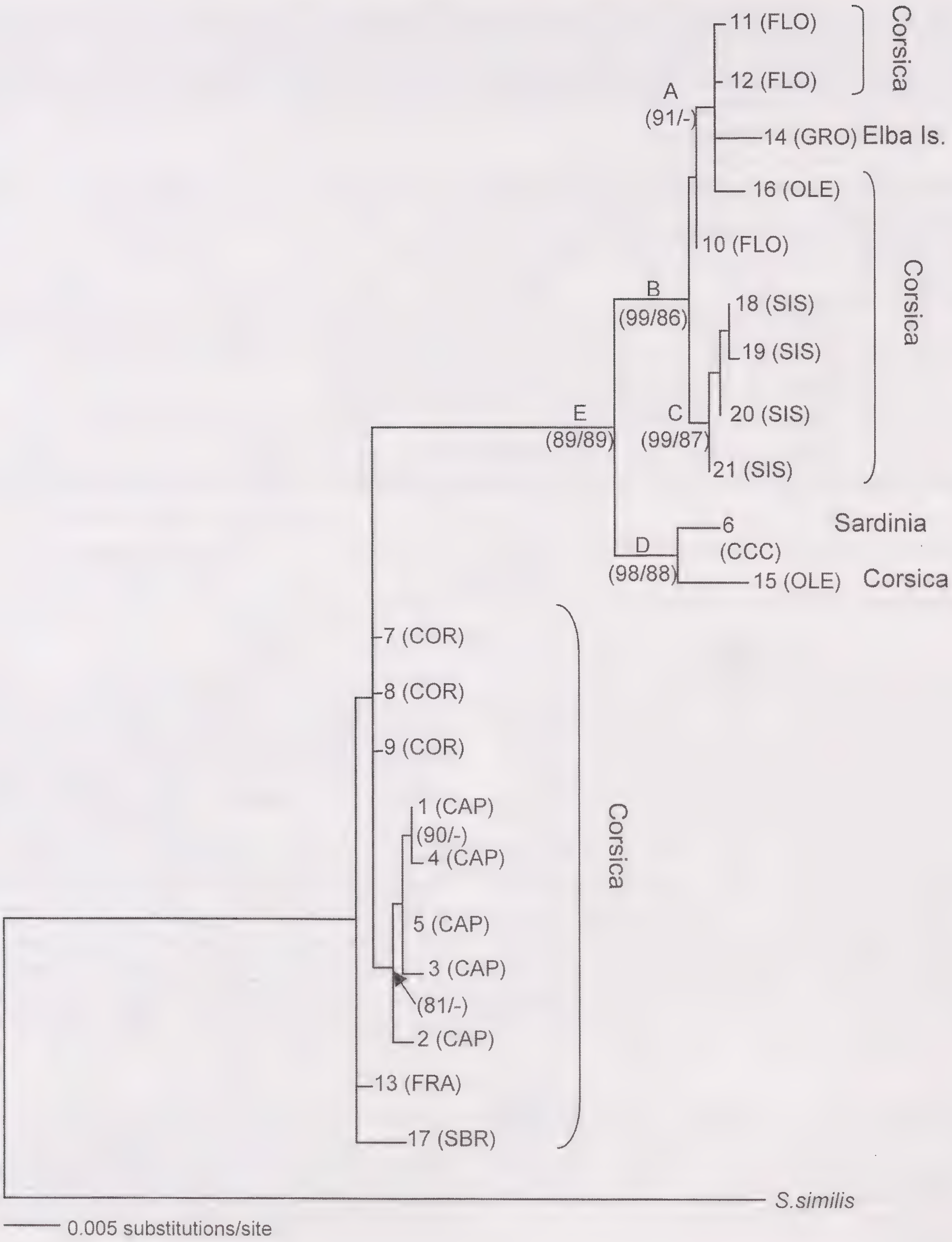

FIG. 2. Bayesian haplotype phylogram based on the GTR+I model of sequence evolution. Only statistical supports ≥ 75% for both the Bayesian (2,000,000 generations) and NJ searches (1000 bootstrap replicates) are reported on branches (first and second values). Population codes are as in Table 1 while haplotype numbers are as in Table 2. A, B, C, D and E identify nodes for which estimates of divergence times have been calculated (Table 3).

monophyletic clusters. Rather, haplotype 10 (FLO) grouped with one of the two haplotypes found at OLE (16) plus the haplotype sampled on Elba Island (GRO). The second haplotype from OLE (15) was sister to haplotype 6 found on Sardinia. The second group only included Corsican haplotypes. Relationships within this clade were poorly resolved, with only a low support (81%) for haplotypes from CAP as monophyletic in the Bayesian analysis.

TABLE 3. Inferred divergence times (Myr) based on the COI and 16S data partitions and on a variety of published rates for mollusks for supported splits in the *Solatopupa guidoni* haplotype tree of Fig. 2 (nodes A-E). Maximum Likelihood distances (D_{ML}) are based on the models of sequence evolution that best fitted the two data partitions (models selected with MODELTEST). *Rates from Ozawa & Okamoto, 1993; Rumbak et al., 1994; and Marko, 2002. **Substitution models are GTR + I and F81 for COI and 16S data partitions, respectively.

Data partitions/ Rates* (%/Myr)	COI 0.67–1.08		16S 0.5–0.6	
Node	D_{ML}**	Myr	D_{ML}**	Myr
A	0.007 ± 0.002	0.52 ± 0.14–0.32 ± 0.009	0.0005 ± 0.0005	0.05 ± 0.05–0.04 ± 0.04
B	0.01 ± 0.001	0.75 ± 0.07–0.46 ± 0.046	0.0012 ± 0.001	0.12 ± 0.100–0.1 ± 0.083
C	0.001 ± 0.0006	0.07 ± 0.044–0.05 ± 0.027	0.000	-
D	0.015	1.12–0.69	0.009	0.9–0.75
E	0.034 ± 0.001	2.53 ± 0.07–1.57 ± 0.046	0.004 ± 0.0004	0.4 ± 0.03–0.33 ± 0.03

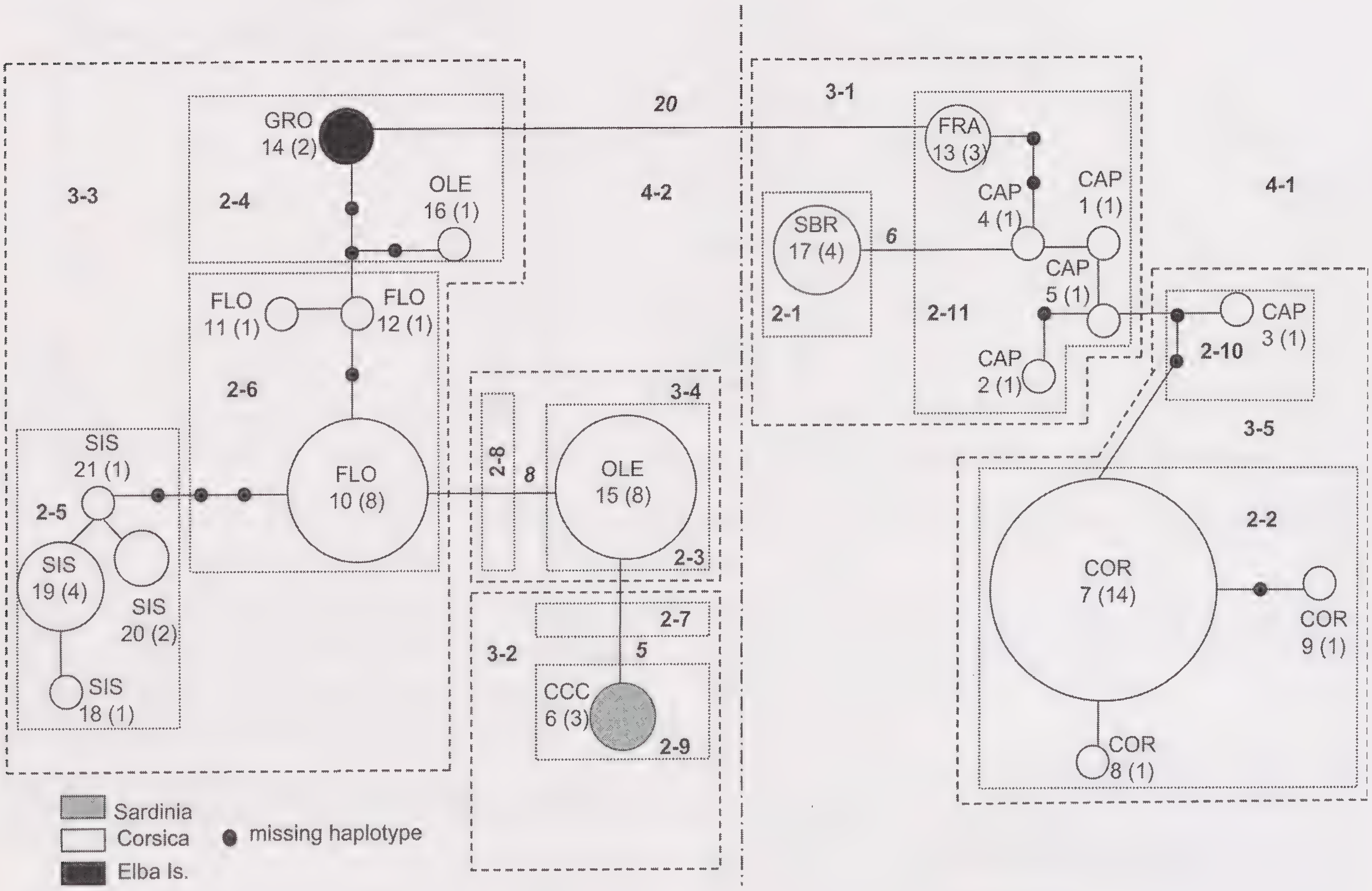

FIG. 3. Haplotype network and Nested Clade Phylogeographical Analysis of *Solatopupa guidoni* populations derived from COI + 16S data. The relative size of the circles is proportional to the number of individuals carrying that particular haplotype; different shadings indicate the geographic origin of haplotypes. Coding of populations and numbering of haplotypes are as in Tables 1 and 2. Numbers in parentheses indicate how many individuals carried that particular haplotype. Black dots are missing haplotype. Haplotypes are always one mutational step away from each other if not indicated otherwise (i.e. when the number of steps was higher than 4 numbers in italics are the mutational steps necessary to connect haplotypes; missing haplotypes are not represented in these particular circumstances). The Figure also shows the nesting design as obtained with ANeCA. Clades are designed as *x-y*, where *x* represents the nesting level (from the haplotype to the 4th level but haplotype and 1-step levels are not shown) and *y* is the number assigned to that particular clade.

TABLE 4. Nested clade analysis of the geographical distribution of *Solatopupa guidoni* haplotypes. Only the six clades with significant GEODIS results are shown (see *P* value of the χ^2 test; third column). Clade names and nesting design are as in Fig. 3; the geographical distribution of clades is also shown. The last two columns show the sequence followed in the inference key implemented in ANeCA and the resulting biological inference.

Clade	Distribution	*P*	Inference key sequence	Biological inference
2-11	FRA, CAP (Corsica)	0.050	1,2, tip-interior status cannot be determined	Inconclusive outcome
3-1	SBR, FRA, CAP (Corsica)	0.005	1,19, no	Allopatric fragmentation
3-3	OLE, FLO, SIS, GRO (Corsica, Elba Is.)	0.000	1,2,11,12, no	Contiguous range expansion
4-1	COR, SBR, FRA, CAP (Corsica)	0.000	1,2, tip-interior status cannot be determined	Inconclusive outcome
4-2	CCC, OLE, FLO, SIS, GRO (Sardinia, Corsica, Elba Is.)	0.000	1,2,3,4,9, no	Allopatric fragmentation
Total	All populations (Sardinia, Corsica, Elba Is.)	0.000	1,2, tip-interior status cannot be determined	Inconclusive outcome

Because the Corsican populations did not form a monophyletic group, we tested this alternative hypothesis against the unconstrained topology presented in Figure 2. Yet, the AU test rejected the monophyly of the Corsican populations ($P = 0.008$).

The LRT test conducted on trees with and without the molecular clock alternatively relaxed and enforced could not reject the hypothesis of homogeneous rates along the branches of our phylogeny [$-\ln L$noclock = 2114.27; $-\ln L$clock = 2115.13; $2\Delta L = 1.72$; degrees of freedom = 20; critical χ^2 value ($P = 0.05$) for 20 degrees of freedom = 31.41]. We could therefore calculate divergence times for labeled nodes in the tree of Figure 2 (A to E); the results of these calculations are presented in Table 3. These estimates have to be considered cautiously, as not all the above nodes were unambiguously supported by our phylogenetic searches though the network analysis retrieved almost identical groupings. Time estimates based on COI ranged in age from 0.05 ± 0.027 Myr (node C) to 2.53 ± 0.07 Myr (node E); those based on 16S varied between 0.04 ± 0.04 Myr (node A) and 0.4 ± 0.03 Myr (node E).

TABLE 5. Results from the analysis of molecular variance (AMOVA). Populations have been grouped alternatively into three (scheme I) or four groups (scheme II). Scheme I corresponds to the three islands (Sardinia, Corsica and Elba Island) sampled for the study while in scheme II we split Corsican haplotypes into two groups (COR, SBR, FRA, CAP and OLE, FLO, SIS, respectively), as suggested by phylogenetic and network analyses (Figs. 2, 3).

Analysis structure	Source of variation	Sum of squares (d.f.)	Variance component	P	Fixation index	% of variation
Scheme I	Among groups	2.68 (2)	Va = 0.062	0.040	$\Phi_{CT} = 0.113$	11.35
	Among populations	15.01 (6)	Vb = 0.310	0.000	$\Phi_{SC} = 0.636$	56.44
	Within populations	9.02 (51)	Vc = 0.180	0.000	$\Phi_{ST} = 0.677$	32.21
Scheme II	Among groups	6.12 (3)	Va = 0.023	0.000	$\Phi_{CT} = 0.047$	8.94
	Among populations	11.56 (5)	Vb = 0.305	0.000	$\Phi_{SC} = 0.633$	63.32
	Within populations	9.02 (51)	Vc = 0.176	0.000	$\Phi_{ST} = 0.650$	27.74

TABLE 6. F_{ST} values among the *Solatopupa guidoni* populations included in the study. Bold numbers indicate statistical significance after sequential Bonferroni correction for multiple comparisons (significance assessed by 1,000 permutations in ARLEQUIN 3.01).

	Sardinia				Corsica				Elba Is.
Population	1	2	3	4	5	6	7	8	9
1-CCC	-								
2-COR	**0.798**	-							
3-SBR	**1.000**	**0.809**	-						
4-FRA	**1.000**	**0.798**	**1.000**	-					
5-CAP	**0.387**	**0.519**	0.452	**0.387**	-				
6-OLE	**0.828**	**0.765**	0.843	**0.828**	**0.469**	-			
7-FLO	0.711	**0.703**	**0.733**	0.711	0.377	**0.696**	-		
8-SIS	**0.479**	**0.563**	**0.522**	**0.479**	0.138	**0.524**	0.449	-	
9-GRO	**1.000**	**0.784**	**1.000**	**1.000**	0.285	**0.809**	**0.681**	0.415	-

Nested Clade Phylogeographical Analysis, AMOVA and F_{ST} values

Figure 3 illustrates the network obtained with TCS (Clement et al., 2000) and shows the nesting design with four nested levels. The pattern of relationships in the network was generally congruent with that depicted by the phylogenetic tree of Figure 2. Clades 4-1 and 4-2 corresponded to the two major groups of haplotyes found in the phylogenetic searches. These clades were separated by a deep phy-

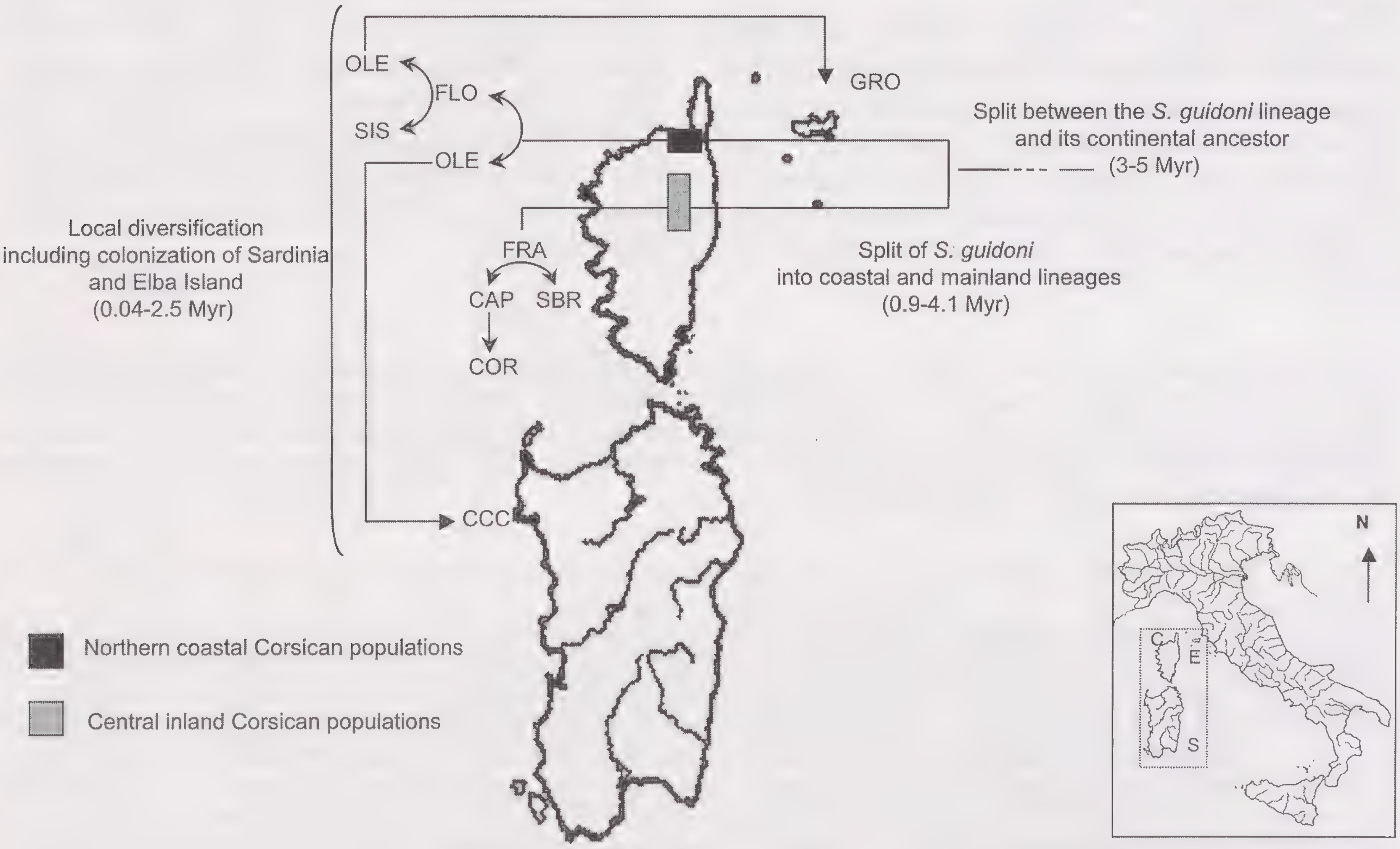
OLE
FLO
SIS
OLE
GRO
Split between the S. guidoni lineage and its continental ancestor (3-5 Myr)
Local diversification including colonization of Sardinia and Elba Island (0.04-2.5 Myr)
FRA
CAP
SBR
COR
Split of S. guidoni into coastal and mainland lineages (0.9-4.1 Myr)
CCC
N
C
E
S
■ Northern coastal Corsican populations
▨ Central inland Corsican populations

FIG. 4. Schematic of proposed phylogeographic history of *Solatopupa guidoni* with patterns and timing of the major events of differentiation within the species as inferred from phylogenetic and nested clade analyses combined with molecular clock estimates. See text for detailed discussion.

logeographic break of 20 substitutions. The two haplotypes from OLE (15 and 16) were not linked to each other. Overall, the network analysis confirmed that the Corsican haplotypes do not form a monophyletic entity and can be split into two major sub-groups. The nesting procedure produced eight clades that could be tested for geographical association, and six of them resulted in significant permutational contingency tests indicating a non-random geographical distribution of haplotypes (Table 4). The inferences derived from Templeton's keys suggested allopatric fragmentation for clades 3-1 and 4-2 and contiguous range expansion for clade 3-3. In three circumstances (2-11, 4-1, and for the total cladogram) the inference keys gave inconclusive outcomes because of the unresolved status of tip-interior position of the clades.

Hierarchical analysis of molecular variance (AMOVA) is reported in Table 5. Pooling Corsican populations in a single group or splitting them into two groups did not change the main outcomes of the analysis. AMOVA showed that most genetic heterogeneity was apportioned among populations within groups and within populations. Together these two levels explain between 87% and 92% of the overall variation, depending on the hierarchical scheme adopted (I or II; Table 5). These results, along with those of the pairwise F_{ST} analysis reported in Table 6, indicate a general high level of genetic differentiation. The vast majority of pairwise F_{ST} values were over 40% and statistically significant after sequential Bonferroni correction.

DISCUSSION

The main findings of this study can be summarized as follows: *S. guidoni* is a genetically structured species that can be broadly divided into two lineages. One of these lineages is limited to central inland Corsica, while the second includes northern coastal Corsica sites plus Sardinia and Elba populations. Patterns of relationships suggest that differentiation within Corsica predated between-island divergence. This translates into a lack of reciprocal monophyly of lineages occurring on the three landmasses. While Sardinia and Elba both harbor monophyletic (monotypic) lineages (but a very limited number of individuals were analyzed for these locations), they are nested in one of the Corsican clades.

Levels of Divergence and Population Structure

Numerous studies have revealed a broad range of intraspecific mtDNA differentiation in terrestrial mollusks (2.9–30% of sequence divergence) (Thomaz et al., 1996; Pfenninger & Magnin, 2001; Davison, 2002; Holland & Hadfield, 2002; Uit de Weerd et al., 2005). We found an average level of sequence divergence of 3.6 ± 0.2% between the two alleged haplo-groups of *S. guidoni*. This is close to the lower bound of the aforementioned range of mtDNA variation. These results suggest that processes of differentiation within the species are relatively recent. Our molecular clock estimates below support this hypothesis. In addition, Giusti (1977) stressed that there are no clear differences in shell morphology or internal anatomy that could help distinguish Sardinian, Corsican, and Elba Island populations from one another. Thus, neither genetics nor morphology (Giusti, 1977) support the need for taxonomic revision of the species, contrary to the claim of Boato et al. (1985).

On the other hand, our data indicate a general lack of gene flow among populations of *S. guidoni*, as attested by haplotype frequencies, AMOVA results and generally high and significant pairwise F_{ST} values. AMOVA showed that populations were significantly differentiated at all three levels of the hierarchical design, with a large fraction of variation apportioned among populations, whatever hierarchical scheme was adopted in the analysis (Table 5). These results are in line with allozyme data that demonstrated a substantial lack of gene flow in the species, with populations bearing a considerable number of private and fixed alternative alleles and with an average number of migrants per generation among locations of 0.036 (Boato, 1988). Concordant patterns in markers with different characteristics (nuclear vs. mitochondrial) suggest the same underlying evolutionary scenario. These results also fit well with some general characteristics of land snails. Populations of these organisms are often divided into small breeding units (demes) that are partially, if not completely, isolated from one another by habitat discontinuities. Movements of individuals between demes are very rare, since distance between nearby demes is usually larger than the average dispersal range of individuals (Fiorentino et al., 2009, and references therein). It is therefore not surprising that most of the molecular papers published so far on terrestrial mollusks have revealed considerable population structuring and geographic variation (reviewed in Davison, 2002). Stochastic processes (i.e., genetic drift and founder effect) have been identified as the prominent factors shaping genetic traits of populations. Coalescence of haplotypes is another process that might account for population division in terrestrial mollusks. Their populations are typically arranged in a stepping-stone fashion and divided into discrete demes of large size (hundreds or thousands of individuals). Their high density and the limited migration of individuals between them theoretically translate into much longer persistence (time to common ancestor) of mitochondrial haplotypes with retention of ancient lineages and thus into an increase in overall divergence (Thomaz et al., 1996). It must be noted, however, that population size in *Solatopupa* tends to be small compared to that of other snails and occurrence of demes is scattered (Boato, 1988). These seem to be common traits in rock-dwelling species bound to calcareous substrata (Fiorentino et al., 2009). Moreover, rocks of different geological nature surround the limestone occupied by the species constituting a major impediment to dispersal. Assuming, contrary to what is commonly reported for other land snails, that *S. guidoni* populations are arranged in scattered demes of small size, we argue that time to coalescence may be relatively short, explaining the rather shallow level of divergence.

Historical Biogeography

A number of molecular studies on a taxonomically diverse array of organisms endemic to the peri-Tyrrhenian area have been carried out in the last decade. The organisms include newts, subterranean terrestrial and aquatic isopods, stoneflies, and flightless beetles (Cobolli Sbordoni et al., 1995; Caccone et al., 1997; Ketmaier et al., 1999, 2000, 2003a, b; Fochetti et al., 2004). All these organisms are found in Sardinia and Corsica and some of them also occur in the Tuscan archipelago or coastal areas of Tuscany, that is, the terrestrial and aquatic isopod genera *Oritoniscus* and *Stenasellus* (Cobolli Sbordoni et al., 1995; Ketmaier et al., 1999, 2000, 2003a). In all circumstances, phylogenetic analyses were concordant in sorting Sardinian and Corsican taxa into distinct groups separated by large mutational distances. Tuscan representatives, if any, were always more closely related to the Corsican than to the Sardinian taxa (Cobolli Sbordoni et al., 1995; Ketmaier et al., 1999, 2000, 2003a). Interestingly, a major phylogeographic break between Corsica and Sardinia and a closer affinity between populations from Corsica and the Tuscan archipelago has also been found in plants belonging to the genus *Borago* (Selvi et al., 2006). Conversely, we did not find reciprocally monophyletic lineages in *S. guidoni* on the three islands.

Given the lack of fossil evidence for *S. guidoni*, which prevents the use of tree-based estimates of time divergences (Sanderson, 1997, 2002, 2003), we could only rely on rates published for mollusks. It is important to stress that mtDNA substitution rates are a notoriously difficult issue in gastropods because of their extensively documented accelerated rates of evolution (Chiba, 1999; Pinceel et al., 2005). However, we tend to reject this hypothesis for *S. guidoni* on the grounds of the available allozyme data (Boato, 1988). MtDNA and allozymes support concordant patterns of relationships as shown here. According to Pinceel et al. (2005), this is good evidence that mt- and nuclear DNA rates are not strongly decoupled with mtDNA evolving much faster than nucDNA. If they were, we would have observed divergence in the mtDNA but sharing of alleles at the allozyme loci across locations, which is not the case here as different populations are characterized by a high percentage of private alleles (12.5%; Boato, 1988). Additionally, time estimates for the statistically supported nodes in our phylogeny are all relatively recent, not older than 2.5 Myr. While these are only crude estimates of minimum time since divergence and are based on a not fully supported phylogeny, they are in line with the time frame of genus cladogenesis, which took place between the middle Miocene and the middle-late Pliocene (Ketmaier et al., 2006).

Previous allozyme and DNA sequence data had already suggested that Corsican populations might be divided into a central inland and a northern coastal clade (Boato, 1988; Ketmaier et al., 2006). These would correspond to clades 4-1 and 4-2 of our study (Fig. 3). Sardinian and Elba Island haplotypes are linked to the northern coastal clade. Boato (1988) found a sister relationship between a population sampled close to OLE (Col de Teghine, black dot in Fig. 1) and CCC (same locality as in the present study), but no samples from Elba Island were included in that study. In our previous paper, we retrieved a sister relationship between CCC and GRO, but only analyzed two mainland Corsican populations (FRA and SBR) (Ketmaier et al., 2006). The occurrence of the species on Sardinia and Elba Island can be explained in the light of the paleogeographic history of these landmasses and their connections to Corsica. According to Steininger & Rögl (1984), Corsica and Sardinia became permanently isolated in the Pliocene (5 to 3 Myr BP). More recent data, however, suggests that this is an over-simplified scenario and that multiple relatively short-lived connections between the two islands existed during the Quaternary, up to 20,000 years ago at each glacial peak, as a consequence of the worldwide drop in sea level (Meulenkamp & Sissingh, 2003; Pascucci, per.com.). Our time estimates for the split between the Sardinian population and its closest Corsican relative (haplotype 15, OLE) range from 0.7 to 1.12 Myr BP, depending on the gene used and the substitution rate adopted (Table 3). Although these datings span a relatively broad time scale, they overlap remarkably with the Günz glaciation (West, 1977). They suggest that a (possibly single) dispersal event of the species from Corsica to Sardinia took place at that time, but not during more recent Quaternary connections between the two islands. This hypothesis is further supported by the fact that in Sardinia the species is limited to two nearby locations (Fig. 1), despite the extensive occurrence of limestone throughout the island. The haplotype sampled on Elba Island is deeply embedded within Corsican haplotypes in the phylogenetic tree (Fig. 2), while it has a tip position in the haplotype network (Fig. 3). According to our time estimates, differentiation within this haplogroup occurred in the Pleistocene, between 0.04 and 0.52 Myr BP. Anti-clockwise rotation of the Sardinia-Corsica microplate caused uplifting of coastal Tuscany, including the Tuscan archipelago, which has been repeatedly connected to Corsica as well as to coastal Tuscany during the Quaternary (Lipparini, 1976; Cherchi & Montadert, 1982; Burgassi et al., 1983; Pascucci et al., 1999). As a consequence, the archipelago hosts a fauna transitional between the two areas. The incidence of continental Tuscan and Corsican species in the Tuscan archipelago is directly related to the present-day distance of the different islands from the two source areas, with Elba Island playing the role of stepping stone (Dapporto et al., 2007, and references therein). A close phyletic relationship between Corsican and insular Tuscan species has frequently been found for species with poor dispersal capacities, such as stygobiont taxa (Ketmaier et al., 2003a). This led to the recognition of a Corsica-Sardinian stygofaunal province that also includes the insular areas of Tuscany (Pesce, 1985). We conclude that the most parsimonious scenario to account for the exclusive occurrence of the species on Elba Island (and not on any other island of the Tuscan Archipelago) is based on dispersal. Our time estimate for this event suggests that the species reached Elba Island more recently than Sardinia.

The Phylogeographic Scenario

The nested clade analysis suggests a dispersal event between Corsica and Elba Island with contiguous range expansion (clade 3-3; Fig. 3). This implies movements of individuals as connections between landmasses became available. For clades 3-1 (central inland Corsican lineage) and 4-2 (Sardinia, coastal northern Corsica and Elba Island), the analysis indicated allopatric fragmentation as the most likely biological inference. A combination of vicariance and dispersal events would therefore most effectively explain the current distribution of the species. Phylogeographic relationships within *S. guidoni* are evidently complex; Figure 4 summarizes the following discussion. Based on our phylogenetic, nested clade and molecular clock analyses we propose that immediately after the split from its continental sister taxon, *S. guidoni* underwent further division into a coastal northern and central inland Corsican lineage. Diversification within the latter group is presumably allopatric and associated with the scattered distribution of suitable calcareous rocks among geologically different substrata. Relationships within the coastal lineage are less clear. A first split separated FLO from OLE (haplotype 15, the most abundant in the latter population). The occurrence of the species in Sardinia is difficult to explain because of the large geographical gap separating the Sardinian population from its closest extant population (OLE). In addition, *S. guidoni* has never been found in southern Corsica, despite extensive surveys in the only area with limestone (Bonifacio; shaded in grey in Fig. 1). We cannot exclude that *S. guidoni* had a broader range, occurred there sometime in the past and then became extinct; unfortunately, this is a hypothesis that currently cannot be tested. According to the haplotype network (Fig. 3), FLO gave rise to SIS, probably through a single founder event, as supported by the monophyly of SIS (Figs. 2, 3). Nearly coeval with this was the origin of the lineage that includes the second haplotype found at OLE (haplotype 16) and the haplotype found on Elba Island. The occurrence of divergent haplotypes at OLE implies admixture between presumably allopatrically evolved haplotypes (Avise, 2000). The only known population in the area that we were not able to sample is Col de Teghine (Fig. 1). This population (but not OLE) was included in the allozymic survey of Boato (1988) and it showed affinity with FLO, while bearing private alleles. We tentatively conclude that at OLE a more divergent haplotype (15) met haplotype 16 of more recent origin.

This study reveals that within-island divergence in *S. guidoni* is slightly older than between-island differentiation and that Corsica (the most intensively sampled island) does not harbor a monophyletic lineage. While very little can be derived for Sardinia and Elba Island because of the small sample sizes, for Corsica our results do not support a one island-one clade association, even in an organism with theoretical poor dispersal capacity and distributed over biogeographically well-known areas.

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